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## ORIGINAL ARTICLE

# Clinical and bacteriological characteristics of *Klebsiella pneumoniae* causing liver abscess with less frequently observed multi-locus sequences type, ST163, from Singapore and Missouri, US

Getahun Abate <sup>a</sup>, Tse-Hsien Koh <sup>b</sup>, Morey Gardner <sup>a</sup>, L. Kristopher Siu <sup>c,d,\*</sup><sup>a</sup> Department of Internal Medicine, St. Mary's Health Center, St Louis, MO, United States<sup>b</sup> Department of Pathology, Singapore General Hospital, Singapore<sup>c</sup> Division of Infectious Diseases, National Health Research Institutes, Taiwan<sup>d</sup> Graduate Institute of Basic Medical Science, China Medical University, Taichung, Taiwan

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**KEYWORDS***K. pneumoniae*;  
Liver abscess;  
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type (MLST)

**Background:** *Klebsiella pneumoniae* is the major cause of liver abscesses in several Asian countries. Differences in the type of circulating *Klebsiella* strains and/or the genetic make up of the host seem to be plausible explanations for this.

**Methods:** Two recent *K. pneumoniae* strains isolated from patients with liver abscess, one from Missouri in the US, and a second one from Singapore, were fully characterized by molecular typing, association of virulent genes, neutrophil phagocytosis, susceptibility to serum killing, and lethality in mice.

**Results:** Both strains had mucoid colony morphology and were similar in multilocus sequence type (ST-163), drug-susceptibility profile, resistance to phagocytosis and susceptibility to serum killing. Although ST-163 is a single nucleotide variant (SNV) to the major ST-23, which is specific to serotype K1 *K. pneumoniae* that causes liver abscess in Taiwan, these two isolates differ in capsular serotype. One was serotype K1 and the other K29. Since a serotype K35 with ST163 was reported previously to cause peritonitis, serotype K29 with SNV to ST-23 was not impossible. Pulsed field gel electrophoresis by XbaI digestion showed different restriction patterns. The virulence-associated genes *rmpA* and *aerobactin* were only present in the serotype K1 isolate from Singapore and not in the serotype K29 isolate from Missouri. The serotype K1 isolate was also more virulent to mice.

\* Corresponding author. Division of Infectious Diseases, National Health Research Institutes, 350, Taiwan.  
E-mail address: [lksiu@nhri.org.tw](mailto:lksiu@nhri.org.tw) (L.K. Siu).

**Conclusion:** The reasons underlying the high prevalence of ST-23 or its SNV in *K. pneumoniae* liver abscesses is worth further investigation.

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## Introduction

*Klebsiella pneumoniae* is one of the major causes of pyogenic liver abscess. In Taiwan, over 80% of bacterial liver abscesses are caused by *K. pneumoniae*.<sup>1</sup> In Singapore and South Korea, similar observations have been made.<sup>2,3</sup> Most patients with a *K. pneumoniae* liver abscess display bacteremia and septic metastatic complications, including endophthalmitis and meningitis. Comorbid conditions such as diabetes mellitus and alcoholism are associated with increased risk of *K. pneumoniae* liver abscess.<sup>1,2,4</sup>

Among reports of *K. pneumoniae* liver abscess, the vast majority of patients are Asian. Case reports from other countries including the US have indicated that most patients with *K. pneumoniae* liver abscess were of Asian descent.<sup>5</sup> However, it is not known whether these *Klebsiella* isolates from Asians living abroad are similar to isolates predominantly circulating in Asia. In Asia, serotype K1 is the majority capsular serotype in patients with liver abscess, followed by serotype K2.<sup>1–3</sup> Other virulence-associated genes such as *rmpA* and *aerobactin*, are common in isolates.<sup>6</sup> Turton et al.<sup>7</sup> found that sequence type ST23 was the major sequence type (ST) in *K. pneumoniae* liver abscess isolates and these are specifically associated with serotype K1. To our knowledge, there are no similar studies on the detailed characterization of *K. pneumoniae* isolates from liver abscess patients from countries other than those in Asia. This could be partly because of the very limited number of cases in other countries. This study was conducted to fully characterize two recent isolates from the US and Singapore based on clinical as well as bacteriological features including serotyping, antimicrobial susceptibility, genotyping, virulence evaluation and 50% lethal dose (LD<sub>50</sub>) in mice.

## Methods and materials

### Selected *K. pneumoniae* liver abscess isolates

The isolates for this study were selected by an international collaboration group for *K. pneumoniae* liver abscess study. Liver abscess was diagnosed by ultrasonography or computed tomography (CT) and confirmed by culture. Identification of the isolates was according to standard clinical microbiologic methods. The two isolates in this study were obtained from Singapore and the US. One hypervirulence *K. pneumoniae* liver abscess isolate with serotype K1 from Taiwan<sup>8</sup> was included as a control for virulence assessment.

### Serotyping

Isolates were serotyped using capsule swelling reaction with antisera obtained from the Health Protection Agency

in the UK. In addition, one tube multiplex polymerase chain reaction (PCR) for K1, K2 and K5 was performed according to a previously published method.<sup>9</sup>

### Antimicrobial susceptibility

Antimicrobial susceptibility was determined by microbroth dilution and disk diffusion according to the Clinical and Laboratory Standards Institute (CLSI) method. The following antimicrobial agents were used: ampicillin, cefazolin, cephalothin, amoxicillin/clavulanic acid, cefoxitin, cefotaxime, ceftazidime, aztreonam, imipenem, amikacin, gentamicin, ciprofloxacin and trimethoprim/sulfamethoxazole.<sup>10</sup> All drugs were incorporated into the Mueller-Hinton broth (TREK Diagnostic System Ltd, West Sussex, UK) in serial twofold concentrations from 0.025 to 64 mg/L. Two control strains, *Escherichia coli* ATCC 35218 and ATCC 25922, were included in each test run. Inoculated plates were incubated at 35 °C for 16 to 18 h. The minimal inhibitory concentration (MIC) of each antimicrobial agent was defined as the lowest concentration that inhibited visible growth of the organism.

### PCR for *rmpA* and *aerobactin* genes

PCR was used to determine the presence of the *rmpA* and *aerobactin* genes.<sup>6</sup> An overnight-cultured bacterial colony was added to 300 µl of water and boiled for 15 min to release the DNA template. Previously published primers used for PCR are listed in Table 1. The reaction mixture was kept at 95 °C for 5 min, followed by 40 temperature cycles of 95 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min, and 72 °C for 7 min. The expected PCR product of *rmpA* was 535 bp in length.

### Multilocus sequence typing (MLST)

Multilocus sequence typing was performed according to Turton et al.<sup>7</sup> Sequences of seven housekeeping genes were obtained for isolates from liver abscess patients and carriers. Sequence information was compared with that on the MLST web site ([www.pasteur.fr/mlst/](http://www.pasteur.fr/mlst/)) developed by Keith Jolley. Alleles and STs were assigned accordingly. Sequences of any alleles that were not on the database were submitted to the curator and new allele numbers were obtained. Strains with a difference in two or more alleles were considered to be unrelated.

### Pulsed field gel electrophoresis (PFGE)

Total DNA was prepared and PFGE was performed as described previously.<sup>3</sup> The restriction enzyme *Xba*I (New England Biolabs, Beverly, MA, USA) was used at the temperature suggested by the manufacturer. Restriction fragments were separated by PFGE in 1% agarose gel

**Table 1** Bacteriological characteristics of liver abscess isolate in this study

Isolate	Specimen type	Serotype	Virulence associated gene		MLST							
			RmpA	aerobactin	ST	Allelic profiles						
						gapA	inf	mdh	pgi	pho	rpoB	tonB
US-1	Liver aspirate	K29	—	—	163	2	1	1	1	9	1	12
SG-1	Blood	K1	+	+	163	2	1	1	1	9	1	12

(Bio-Rad, Hercules, CA, USA) in  $0.5 \times$  TBE buffer (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA, pH 8.0) for 22 h at 200 V at 14°C, with ramped times of 2 to 40 s using the Bio-Rad CHEF MAPPER apparatus (Bio-Rad Laboratories, Richmond, CA, USA). Gels were then stained with ethidium bromide and photographed under ultraviolet light. The resulting genomic DNA profiles, or “fingerprints”, were interpreted according to established guidelines.<sup>11</sup>

### Virulence assessment by neutrophil phagocytosis and serum-resistance assay

A neutrophil phagocytosis assay was performed as described previously.<sup>8</sup> Serum bactericidal activity was measured using the method described by Podschun et al.<sup>12</sup> Results were expressed as a percentage of inoculation and responses in terms of viable counts were graded 1 to 6 where:

- Grade 1 is viable counts <10% of the inoculum after 1 and 2 h, and <0.1% after 3 h.
- Grade 2 is viable counts 10–100% of the inoculum after 1 h and <10% after 3 h.
- Grade 3 is viable counts that exceeded those of the inoculum after 1 h, but <100% after 2 and 3 h.
- Grade 4 is viable counts >100% of the inoculum after both 1 and 2 h, but <100% after 3 h.
- Grade 5 is viable counts >100% of the inoculums 1, 2, and 3 h, but that decreased during the third hour.
- Grade 6 is viable counts that exceeded those of the inoculum after 1, 2, and 3 h, and increased throughout this time period.

Each isolate was classified as highly sensitive (grades 1 or 2), intermediately sensitive (grades 3 or 4) or resistant (grades 5 or 6).

### Determination of *K. pneumoniae* dose causing LD<sub>50</sub> in mice

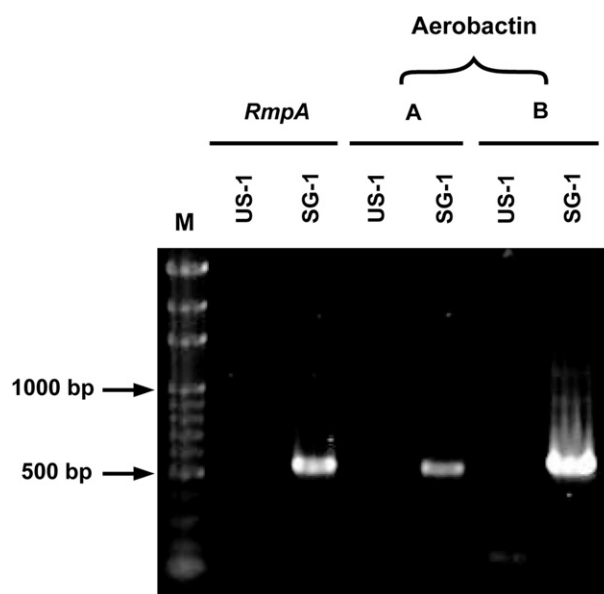
In the determination of LD<sub>50</sub> in mice, 10 mice were used as a sample population for each bacterial concentration. A 10-fold serial dilution of cell-forming unit (cfu) of *K. pneumoniae* was made and BALB/c mice were injected intraperitoneally with 0.1 ml of each concentration. Symptoms and signs of infection were observed for 14 days. Survival of the inoculated mice was recorded and the LD<sub>50</sub> was calculated using SigmaPlot version 7.0 from SPSS Inc. (Chicago, IL).

## Results

### Clinical characteristics of patients selected in this study

Patient 1 was a 53-year-old African American female patient from Missouri, US, who presented with right upper quadrant (RUQ) abdominal pain of 6 weeks' duration with worsening over the 4 days before admission to hospital. The patient had associated nausea, vomiting and fever in the past 4 days. On physical examination, the patient was febrile, tachycardic and had RUQ abdominal tenderness. All other physical findings were negative. On admission, white blood cell count, amylase, lipase and liver enzymes were normal. HIV screening was negative; the antinuclear antibody was negative. Blood culture showed no bacterial growth. CT of the abdomen showed an enlarged liver with clusters of small cystic structures with peripheral edema. Liver biopsy of one of the nodules/cysts revealed pus and culture grew *K. pneumoniae* with a mucoid phenotype.

Patient 2 was a 52-year-old Chinese male from Singapore with a past history of diabetes mellitus and



**Figure 1.** PCR for virulence-associated *rmpA* and *aerobactin* genes. Lanes 1 and 2 were *rmpA* detection for SG-1 and US-1. Lanes 3, 4, 5, and 6 were *aerobactin* detection for SG-1 and US-1 by two different primer sets for gene detection. M = marker.

hypertension on dietary control. He presented with a 5-day history of fever associated with chills and rigors. Two days before admission he also developed nausea, vomiting and RUQ abdominal pain. On examination, the only abnormalities were a fever and mild tenderness in the RUQ. The white blood cell count was elevated at  $12.5 \times 10^9/L$  (86.6% neutrophils), the amylase was 65 U/L and the bilirubin 21  $\mu\text{mol/L}$ . The liver function tests were mildly elevated (aspartate transaminase 83 U/L, alanine transaminase 69 U/L and alkaline phosphatase 106 U/L). A CT scan showed a large (8 cm  $\times$  7.5 cm  $\times$  6 cm) inferior right-lobe liver abscess and gallstones. Blood cultures and aspirate from the liver abscess both grew *K. pneumoniae*.

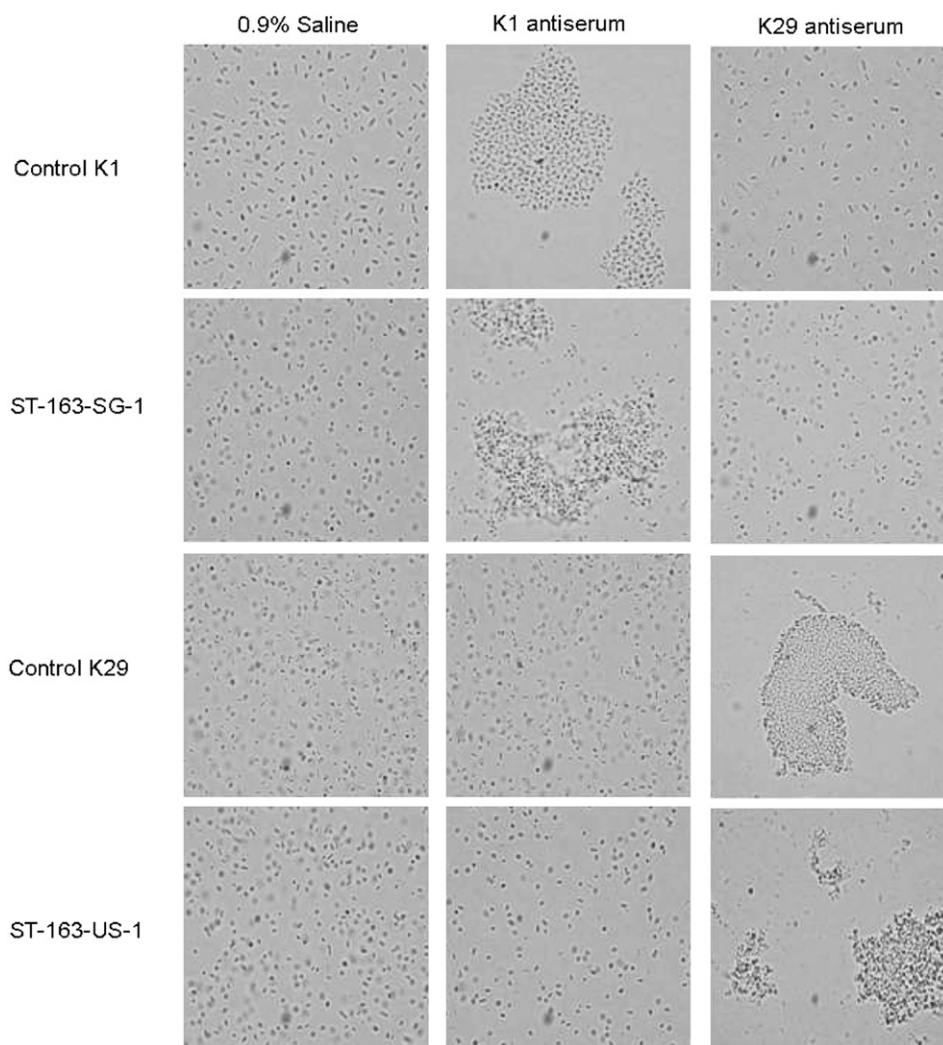
### Bacteriological characteristics of the isolates

Isolates from Missouri and Singapore were designated US-1 and SG-1, respectively. Initially, MLST was performed for US-1 and a search for a matching profile from our MLST database of liver abscess isolates was then performed. Isolate SG-1 was selected for comparison in this study. Both US-1 and SG-1 were of MLST ST 163, having the *gapA*, *infB*,

*mdh*, *pgi*, *phoE*, *rpoB* and *tonB* alleles 2, 1, 1, 1, 9, 1 and 12, respectively (Table 1). ST163 is clonal related to ST23, a major ST type for liver abscess,<sup>7</sup> with the exception of one allelic gene difference by one nucleotide in the *rpoB* gene (A instead of G at nt 130). PCR amplification of virulence associated genes revealed that US-1 was negative for *aerobactin* and *rmpA* while SG-1 was positive for both genes (Fig. 1). PCR for serotyping of K1, K2 and K5 was negative for these three serotypes, while SG-1 was positive for serotype K1 amplification (Table 1). Serotyping by specific anti-serotype K29 and K1 serum confirmed that US-1 and SG-1 were serotype K29 and K1, respectively (Fig. 2).

### Antimicrobial susceptibility and PFGE

US-1 and SG-1 had an identical susceptibility profile, which was intrinsically resistant to ampicillin but susceptible to all antibiotics tested including ampicillin, piperacillin/tazobactam, cefazolin, ceftazidime, cefotaxime, cefepime, aztreonam, imipenem, meropenem, gentamicin, amikacin and ciprofloxacin (Table 2). PFGE showed that US-1 and SG-1 were not genetically related (Fig. 3).



**Figure 2.** Agglutination tests of *Klebsiella pneumoniae*, SG-1 and US-1 strains. Images were photographed using a light microscope (original magnification,  $\times 1,000$ ).



**Table 2** Antimicrobial susceptibility profile for US-1 and SG-1

Antibiotics	Minimal inhibitory concentration ( $\mu\text{g/ml}$ )	
	US-1	SG-1
Ampicillin	$\geq 64$	$\geq 64$
Piperacillin/tazobactam	$\leq 2$	$\leq 2$
Cefazolin	$\leq 4$	$\leq 4$
Cefoxitin	$\leq 4$	$\leq 4$
Ceftazidime	$\leq 0.12$	$\leq 0.12$
Cefotaxime	$\leq 0.12$	$\leq 0.12$
Cefepime	$\leq 0.5$	$\leq 0.5$
Aztreonam	0.5	0.5
Imipenem	$\leq 0.25$	$\leq 0.25$
Meropenem	$\leq 0.25$	$\leq 0.25$
Gentamicin	$\leq 2$	$\leq 2$
Amikacin	2	1
Ciprofloxacin	$\leq 0.5$	$\leq 0.5$

### Neutrophil phagocytosis, susceptibility to serum killing and determination of lethality in mice

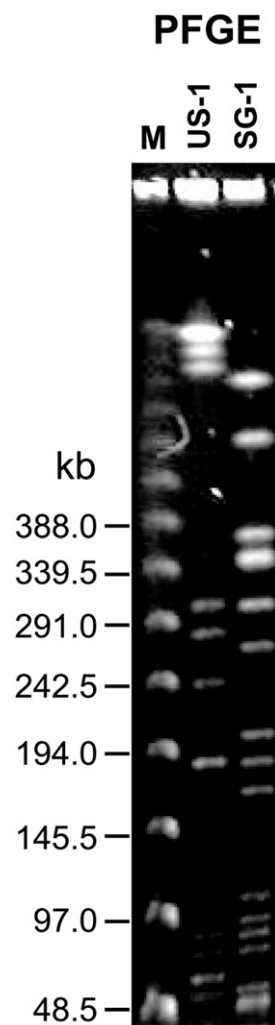
Virulence assessment by neutrophil phagocytosis showed that both isolates were relatively resistant to phagocytosis but susceptible to serum killing (grade 2, see Table 3). Compared with the control isolate, STL-43, and its capsule deficiency strain STL-43 $\Delta rfbP$ , mice lethality assay showed that SG-1 was more virulent ( $3.5 \times 10^5$  cfu) than US-1 ( $>10^6$  cfu) but less virulent than the control STL-43 ( $3.3 \times 10^1$  cfu) liver abscess isolate. The lethality caused by the US-1 isolate was the same as the capsule-deficient STL-43 $\Delta rfbP$  ( $\text{LD}_{50} > 10^6$ ).

### Discussion

*K. pneumoniae* is the major cause of liver abscesses in Taiwan, Singapore and Korea and in Asian descendants living abroad.<sup>13–15</sup> The exact association between this organism and the genetic make up of the host is not yet known. It is clear, however, that the mucoid pattern or capsular phenotype of *K. pneumoniae* plays a role in preventing phagocytosis or intracellular killing.<sup>8</sup> K1 or K2 serotypes cause about 80% of liver abscesses in Taiwan, indicating that these serotypes are the determinants of virulence.<sup>1</sup> Moreover, hypermucoviscosity capsular serotypes K1 and K2 have been shown to be associated with increased virulence and lethality in mice.<sup>15</sup>

There are limited reports of *Klebsiella* liver abscess in the US.<sup>4,14,16–20</sup> In a retrospective study by Rahimian et al on patients admitted with liver abscess,<sup>19</sup> the likelihood of isolating *Klebsiella* was higher in patients of Asian origin. It is not known whether the *Klebsiella* strains circulating in Asian countries are similar to those that are isolated in the US. These limited reports from the US did not include enough characterization of *Klebsiella* isolates to enable a more acceptable comparison with strains circulating in Taiwan and other countries.

We characterized two recent *K. pneumoniae* isolates from the US and Singapore. Both isolates were cultured



**Figure 3.** Pulsed field gel electrophoresis for liver abscess isolates in this study. M = marker; PFGE = pulsed field gel electrophoresis.

from liver abscesses. Both had characteristic mucoid growth and were identical in MLST (ST-163) drug-susceptibility profile as well as resistance to phagocytosis and susceptibility to serum killing. The isolate from Singapore, however, belongs to capsular serotype K1, which is one of the common capsular types in Asia; whereas the isolate from US does not.

*K. pneumoniae* with ST-163 was first described by the Korean Study Group for Liver Abscess.<sup>2</sup> The isolate was shown to be serotype K1 and from liver abscess patients. Subsequently, another *K. pneumoniae* with ST-163 was also identified by a French group.<sup>21</sup> The strain was isolated from a horse in the Netherlands, where it caused peritonitis. This strain was belonged to serotype K35. These results were similar to our cases, which had identical ST-163 but different serotypes.

ST-163 is a single nucleotide variant of ST-23. By comparison, all ST-23 isolates described from liver abscess patients so far have been of serotype K1<sup>7</sup> (our unpublished data). Since the number of clonal complexes of ST-163 isolates with non-serotype K1 is small, it is hard to determine whether the clonal complex including ST-23 can be considered the primary founder for virulent strains. Further

**Table 3** Virulence analysis by combining results obtained from phagocytosis, serum-complement killing and mice lethality

Isolate	Serotype	Phagocytosis <sup>a</sup> (susceptibility)	Serum killing (susceptibility)	LD <sub>50</sub> (cfu) <sup>a</sup>
US-1	K29	28.0 (R)	Grade 2 (S)	>10 <sup>6</sup>
SG-1	K1	15.7 (R)	Grade 2 (S)	3.5 × 10 <sup>3</sup>
STL-43 <sup>b</sup>	K1	34.5 (R)	Grade 5 (R)	33
STL43Δ <i>rfbP</i> <sup>b</sup>	K1	88.4 (S)	Grade 2 (S)	>10 <sup>6</sup>

R = resistance; S, = susceptible. cfu = colony forming unit.

<sup>a</sup> Percentage of *K. pneumoniae* that phagocytosis by neutrophils at 15 min.

<sup>b</sup> Isolates obtained from previous study<sup>22</sup> as a control for virulence assessment. STL-43 was obtained from liver abscess patients and STL43Δ*rfbP* was a capsule deficiency nonpolar-*rfbP* mutant strain derived from STL-43.

analysis should be performed when more non-serotype K1 isolates belonging to the clonal complex, including ST-23, have been collected.

Further characterization showed that the two isolates also differ in *Xba*I restriction patterns. Moreover, the US isolate was far less virulent, while the isolate from Singapore was similar to the control isolate from Taiwan in terms of lethality in mice. These results indicate that despite key similarities, such as liver pathology and mucoid phenotypic pattern, the isolate from the US is different from the *K. pneumoniae* isolate that is predominant in Asia.

The reasons underlying the high prevalence of ST-23 or its single nucleotide variant in *K. pneumoniae* liver abscesses is worth further investigation. Our observations need to be confirmed by comparison with a larger number of isolates.

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